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<p>(54) Title: PRODUCTION OF ANTIBODIES FROM TRANSGENIC ANIMALS (57) Abstract Chimaeric or wholly foreign immunoglobulin is obtained from cells or body fluid of a transgenic animal which has had inserted into its germline genetic material that encodes for at least part of an immunoglobulin of foreign origin or that can rearrange to encode a repertoire of immunoglobulins, i.e. derived from a different animal source. For example, wholly human immunoglobulins may be produced from a transgenic mouse, possibly in response to an immunogen subsequently introduced to the mouse.</p>		

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PRODUCTION OF ANTIBODIES FROM TRANSGENIC ANIMALSField of Invention

This invention concerns the production of antibodies (or immunoglobulins).

Summary of the Invention

According to one aspect of the present invention there is provided a method of producing an immunoglobulin, comprising obtaining immunoglobulin from cells or body fluid of a transgenic animal which has had inserted into its germline genetic material that encodes for at least part of an immunoglobulin of foreign origin or that can rearrange to encode a repertoire of immunoglobulins.

An immunoglobulin of foreign origin means an immunoglobulin derived from a different animal source. For example where the transgenic animal is a mouse, the inserted genetic material is of non-mouse, e.g. human, origin.

The inserted genetic material may be produced from an animal source, or may be produced synthetically. The material may code for at least part of a known immunoglobulin or may be modified to code for at least part of an altered immunoglobulin. Suitable techniques for these processes are well known.

The inserted genetic material may be expressed in the

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transgenic animal, resulting in production of an immunoglobulin derived at least in part from the inserted material. It is found the genetic material is rearranged in the transgenic animal, so that a repertoire of immunoglobulins with part or parts derived from inserted genetic material may be produced even if the inserted genetic material is incorporated in the germline in the wrong position or with the wrong geometry. Depending on the nature of the inserted material, the animal may produce a chimaeric immunoglobulin, e.g. of mixed mouse/human origin, where the genetic material of foreign origin encodes only part of the immunoglobulin, or the animal may produce an entirely foreign immunoglobulin, e.g. of wholly human origin, where the genetic material of foreign origin encodes an entire immunoglobulin. Potentially therapeutically useful immunoglobulins suitable for use with humans thus may be produced by use of the invention.

Polyclonal antisera may be produced from the transgenic animal following immunisation. Alternatively, monoclonal antibodies may be produced from the transgenic animal, eg by fusing spleen cells from the animal with myeloma cells and screening the resulting hybridomas to select those producing the desired antibody. Suitable techniques for such processes are well known to those skilled in the art.

In an alternative approach, the genetic material may be incorporated in the animal in such a way that the desired antibody is produced in body fluids such as serum or external secretions of the animal, such as milk, colostrum or saliva. For example, by inserting in vitro genetic material encoding for at least part of an immunoglobulin

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of foreign origin into a gene of a mammal coding for a milk protein and then introducing the gene to a fertilised egg of the mammal, eg by injection, the egg may develop into an adult female mammal producing milk containing immunoglobulin derived at least in part from the inserted genetic material. The desired antibody may then be harvested from the milk. Suitable techniques for carrying out such processes are known to those skilled in the art.

Another possibility involves removal from the animal of immunoglobulin-producing cells generated by the animal after insertion of genetic material, followed by in vitro selection of cells producing an immunoglobulin of interest. The immunoglobulin can then be produced in vitro from the selected cells in known manner.

It has been found that a transgenic animal can produce chimaeric or foreign immunoglobulin (derived from inserted genetic material) in response to an immunogen subsequently introduced to the animal. Accordingly, by introducing foreign, eg human, genetic material encoding for substantially the entire species specific regions of an immunoglobulin it may be possible to stimulate the animal to produce foreign immunoglobulin to any antigen introduced to the animal. The transgenic animal could thus provide a highly useful, convenient and valuable source of human immunoglobulins to a large range of antigens.

It is though that it may be important for the inserted genetic material to be integrated in proximity genome for successful rearrangement. The inserted genetic material may thus be in the form of DNA cloned into prokaryotic vectors such as plasmids and cosmids. Multiple plasmids

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or cosmids may also be used, but it is probably necessary for these to integrate in proximity on the genome. It may also prove possible to insert larger DNA fragments by using yeast artificial chromosome vectors (see Burke, D T, Carle, G F and Olson, M V (1987) "Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors" *Science*, 236, 806-812), or by introduction of chromosome fragments (see Richer, J and Lo, C W (1989) "Introduction of human DNA into mouse eggs by injection of dissected human chromosome fragments" *Science* 245, 175-177).

The inserted genetic material may be introduced to the host in conventional manner, for example by injection or other procedures into fertilised eggs or embryonic stem cells.

It may be convenient to use a host animal that initially does not carry genetic material encoding immunoglobulin constant regions so that the resulting transgenic animal will use only the inserted foreign genetic material when producing immunoglobulins. This can be achieved either by using a naturally occurring mutant host lacking the relevant genetic material, or by artificially making mutants eg in cell lines ultimately to create a host from which the relevant genetic material has been removed.

Where the host animal carries genetic material encoding immunoglobulin constant regions, the transgenic animal will carry the naturally occurring genetic material and the inserted genetic material and will produce immunoglobulins derived from the naturally occurring genetic material, the inserted genetic material and mixtures of both types of genetic material. In this case the desired immunoglobulin

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can be obtained by screening hybridomas derived from the transgenic animal, eg by exploiting the phenomenon of allelic exclusion of antibody gene expression or differential chromosome loss.

In a further aspect the present invention produces a transgenic animal, particularly a non-human mammal, which has had inserted into its germline genetic material that encodes for at least part of an immunoglobulin of foreign origin, or that can rearrange to encode a repertoire of immunoglobulins.

The invention also includes within its scope an immunoglobulin produced from a transgenic animal in accordance with the invention or produced by the method of the invention.

In one example, lines of transgenic mice were established, carrying a DNA segment introduced into their germline that contains germline-configuration immunoglobulin VH genes, some of the D segments, and all the JH and Cmu gene segments. One of the VH genes, all the JH segments and the exons encoding the secreted heavy-chain constant region of IgM antibody were of human origin, with the remaining material being of mouse origin. The gene segments undergo productive VH-D-JH joining in the lymphoid tissue of the transgenic mice, with resultant synthesis of human/mouse chimaeric IgM antibody in serum.

Following immunisation, hybridomas have been established by fusion between spleen cells from the transgenic mice with the NSO myeloma. Many of the hybrids secrete human chimaeric IgM monoclonal antibodies. These lines of transgenic mice can therefore be used for the production

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of chimaeric human antisera or monoclonal antibodies.

Further, the mice make a response following immunisation with human antigens, producing chimeric antibodies to introduced antigen, and this approach should therefore also prove useful for the production of a repertoire of conventional human or chimaeric human antibodies directed against human as well as heterologous antigens, as the transgenic mice will not be tolerant to human antigenic determinants.

In another example, transgenic mice carrying exclusively human VH, D, JH and C mu sequences were produced by injecting into fertilised mouse eggs cosmid containing human VH genes, D segments, J segments and the C mu constant region. Resulting mice produced between 10 and 100 ug/ml antibody containing human mu chains in their serum, with mouse IgM being present at about 200 ug/ml.

The invention will be further described, by way of illustration, in the following Examples which refer to the accompanying drawings, in which:

Figure 1 illustrates the structure of plasmid DNA;

Figure 2 is a Southern blot analysis of DNA from tissues and from hybridomas derived from transgenic mice with the DNA of Figure 1 incorporated into the germline;

Figure 3 is a Northern blot analysis of cytoplasmic RNA from hybridomas from the transgenic mice;

Figure 4 is an analysis of immunoglobulin secreted by two of the hybridomas established from the transgenic mice;

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Figure 5 illustrates the structure of IgA lambda plasmid DNA;

Figure 6 is a series of FACS histograms showing fluorescence intensity of a fixed number of cells plotted against cell number;

Figure 7 is a series of FACS profiles showing fluorescence intensity of a fixed number of cells plotted against scatter, with each dot representing a cell; and

Figure 8 is a further series of FACS histograms similar to Figure 6.

EXAMPLE 1

The Transgene

Plasmid DNA with the structure shown in Figure 1 was injected into mouse eggs. In this Figure human sequences are represented by filled bars; mouse sequences by unfilled bars; vector by cross-hatched bars, and D elements by reverse hatched bars. Restriction endonuclease cleavage sites are abbreviated as follows: Bg, BglII; P, PvuI; R, EcoRI; K, KpnI; B, BamHI; X, XbaI; H, HindIII.

The provenance of the constituent DNA segments is as follows:

The Vector: The entire "mini-IgH locus" was cloned between the EcoRI and BglII site of a pUC12 derivative that contains BglII linkers cloned into a filled-in NarI

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site.

The VH genes: There are two VH genes in the mini-locus. The VH26 is of human origin and was obtained as a BglII-EcoRI 0.85 Kb fragment from phage lambda VH26 [Matthyssens & Rabbitts (1980) PNAS 77, 6561-6565]. The VH-186.2 is a germline VH gene from C57BL/6 mice and was obtained as 2.5 Kb SacI-KpnI fragment [Bothwell, Paskind, Reth, Imanishi-Kari, Rajewsky & Baltimore (1981) Cell 24, 625-637].

The D segments: the BALB/c mouse D-Q52 element was obtained as a 221 nt XhoI-SacI fragment. Following cloning between the SacI and SalI sites of M13tg131, site directed mutagenesis with either oligonucleotide

5'-GCGTCACCGTGGTAGCTGCTACCGTAGTAATAAACACTGTGGTCC or
5'-GCGTCACCGTGGTCGTAACCATAGTAGACACTGTGGTG

was used to create M13tg131 clones carrying D elements related to those of the mouse SP2 and FL16 families, respectively. These D families are found in both mouse and human. Another D element - the human D-Q52 - was included within the human JH cluster (see below).

The JH cluster: A 3.5 Kb BglII fragment from human DNA was used that includes the six functional human JH segments, three pseudo JHs as well as the human D-Q52 element [Ravetch et al. (1981) Cell 27, 583-591].

The IgH Enhancers: Part of the human IgH enhancer is included within the BglII fragment containing the JH cluster. A full copy of the mouse enhancer is included within the 1 Kb XbaI fragment.

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The Switch region and Cmu region: The 7.5 Kb XbaI fragment of human DNA includes the mu switch region and exons 1 to 4 of the mu heavy-chain constant region. The mu membrane exons and the bulk of the intron between the Cmu4 exon and the CmuM1 membrane exon are provided by a 2.5 Kb HindIII-SphI fragment of the mouse mu CH gene in which the SphI site was converted to a BglII site by use of linkers.

The Transgenic mice

Plasmid DNA was linearised with BglII, purified after electrophoresis in an agarose gel and injected into the male pronucleus of fertilised eggs of C57BL/6J x CBA/Ca mice as previously described [Reik et al. (1987) Eur. J. Immunol. 17, 465-469]. Southern blot analysis of tail DNA revealed that 12 of the 32 mice born carried the mini-locus. Most subsequent work was performed on offspring of three founder mice - H1g 17, 19 and 29 all of which carry a low number (2-5) of copies of the mini-locus.

Serum Assays

Serum of the founder mice was tested by ELISA for the presence of antibody containing antigenic determinants characteristic of human IgM. The unimmunised transgenic mice proved to contain between 10 and 100 ug/ml of chimaeric human IgM in their serum. Immunofluorescence analysis of lymphocytes in peripheral blood also revealed the presence of cells staining with biotinylated species-specific anti-human Ig M antibody and fluorescein-conjugated streptavidin.

Hybridomas from transgenic mice

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Transgenic mice were immunised intraperitoneally with either human red blood cells or sheep red blood cells. Spleens were removed at various times after immunisation, fused with the NSO myeloma and hybrids selected in HAT medium. Many of these hybrids made chimaeric human IgM as revealed by ELISA assay.

DNA Rearrangement of the mini-locus

Southern blot analysis of DNA from tissues from the transgenic mice as well as from the hybridomas revealed that there is a high frequency of DNA rearrangements within the mini-Ig locus in the lymphoid tissue of the transgenic mice.

DNA from tissues or hybridomas established from the transgenic mice was digested with EcoRI and hybridized with a human IgH enhancer probe (BalI-BglII fragment) that hybridizes to the region between the human J6 element and the mouse IgH enhancer in the mini-locus. The results of Southern blot analysis of the DNA are shown in Figure 2. The sizes in Kb of marker fragments are given in the Figure.

Transcription of the mini-locus

Cytoplasmic RNA (5 ug) from the NSO fusion partner or from hybridomas from the transgenic mice was probed with human Cmu, human VH26 or mouse VH186 probes. The results of Northern blot analysis of the cytoplasmic RNA are shown in Figure 3, which reveals that the hybridomas contained mRNA that hybridized with probes for human mu as well as for either or both of the VH26 or VH186 V genes. Thus both the human VH26 and mouse VH186 are able to rearrange and

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thus create a cell-line that secretes a chimaeric human IgM antibody.

Antibody secretion by hybridomas from the transgenic mice

Protein production by cloned hybridoma cell-lines was analysed by use of biosynthetic labelling with L-[³⁵S] methionine and subsequent purification with anti-human mu antiserum. In particular, cells were incubated overnight in medium containing L-[³⁵S] methionine and IgM antibody purified from the culture supernatant by immunoprecipitation and an anti-human mu antiserum. The purification from the supernatants of the transgenic hybridomas 35.5 and 24a was performed in the presence of a large excess (50ug) of non-radioactive, purified mouse monoclonal IgM antibody (B1-8) as indicated by "+" in Figure 4. As seen using the mouse IgM secreting cell-line, the anti-human mu antiserum cross-reacts with mouse mu but this cross-reaction can be competed by non-radioactive mouse B1-8 IgM antibody - see the four lanes on the left of Figure 4.

This illustrates that it is possible to establish hybridomas from these transgenic mice that secrete antibodies with different human mu chains.

EXAMPLE 2

Transgenic animals can be created that produce specific antibodies in both their body fluids and external secretions. By way of illustration, this example concerns transgenic mice that carry integrated into their germline the genes encoding the heavy and light chains of an antigen-specific chimaeric human IgA2 antibody.

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Nine transgenic mouse lines were established that carried germline integrations of the DNA illustrated in Figure 5. In Figure 5, the thick filled lines depict mouse Ig DNA, the hatched lines human DNA, open boxes the mouse IgH and SV 40 enhancers, and thin filled lines the pSV2gpt vector. Restriction site abbreviations are as in Figure 1. The plasmid is a derivative of pSV-VNPH alpha 2 described previously [Bruggemann et al, J. Exp. Med (1987) 166, 1351-1361] and contains a 7.4 kb EcoRI fragment including the rearranged lambda 1 gene of the mouse HOPC2020 plasmacytoma [Bernard et al (1978), Cell 15, 1133-1140]. Plasmid DNA was linearised at the PvuI site in the vector and transgenic mice derived as previously described [Reik et al, Eur. F. Immunol (1987) 17, 465-469].

The transgenic IgA 2 lambda antibody has specificity for the hapten 4-hydroxy-3-nitrophenacetyl (NP). Expression of the transgenic antibody was measured by ELISA assay using NP-bovine serum albumin coupled to the plastic plate and developing with a biotinylated anti-human alpha antiserum. Total Ig was measured using anti-mouse Ig coated plastic and developing with biotinylated anti-mouse kappa antiserum. The concentration of chimaeric anti-NP IgA2 was determined in the colostrum, serum and milk from seven transgenic mice and one control mouse, and the results are given in Table 1. Colostrum was taken from the mother following hormonal injection within 24 hours of giving birth and milk was taken from the mother when the litter was 13-15 days old; serum was obtained at the same time as milk.

From these data it is clear that transgenic animals can be used for specific antibody production thus allowing large

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scale production from milk, colostrum, sera, saliva etc as well as allowing the breeding of animals that yield a milk that is dosed with specific beneficial antibodies. It is also clear that the concentration of transgenic antibody is higher in colostrum than in milk. Moreover, the presence of the transgene does not affect the ability of the animal to make a large amount of endogenous antibody. The animals show no signs of being significantly immunodeficient or unhealthy.

Example 3

In this example, antigen-specific hybridomas were produced using human mu chains from transgenic mice.

To show that the transgenic mice described in Example 1 can be used to produce antigen-specific antibodies in which the heavy-chain contribution to the antigen-combining site is provided by the transgenic human heavy-chain minilocus, the transgenic mice were immunised with 10^7 sheep red cells (SRC). Spleen cells were fused 6 days later with the NSO plasmacytoma (Köhler and Milstein, Nature, 256, 495-497, 1975). Cells were plated out in 96-well costar plates such that the expected seeding frequency was 1 hybridoma per well. Human mu positive hybrids were detected in an ELISA using biotinylated anti-human IgM. Antigen-specific hybrids have been identified in haemagglutination using sheep red cells. Wells were chosen that contained antibodies specific for sheep red cells and that contained human mu but neither mouse mu nor mouse gamma heavy chains as determined by ELISA.

To demonstrate that the antibody secreted by the selected hybridomas was indeed a human mu/mouse light chain anti-

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SRC immunoglobulin, analysis in a fluorescence-activated cell sorter (FACS) was used. For FACS analysis, 10^7 sheep red cells were incubated with 20 μ l culture supernatant for 30 min, washed with phosphate-buffered saline once and further incubated with either biotinylated anti-human μ heavy chain antiserum, biotinylated anti-mouse μ antiserum or biotinylated anti-mouse kappa light chain anti-serum. After 30 min cells were washed as before and incubated with FITC coupled streptavidin. Cells were washed after 30 min and after gentle disruption were ready for the analysis.

Results for 3 different hybrids producing IgM directed against the sheep red cell antigen are shown in the FACS histograms of Figures 6, 7 and 8, respectively.

The letters A, B, C and D in these Figures denote different staining. Figures 6A, 7A, 8A are results for negative controls using sheep red cells alone, sheep red cells incubated with antibody or sheep red cells incubated with fluoresceinated (FITC) second antibody (either anti-human μ anti-mouse μ , anti-mouse kappa). Figures 6B, 7B, 8B are results using sheep red cells incubated with hybrid culture supernatant and fluoresceinated (FITC) anti-human μ . Figures 6C, 7C, 8C are results using sheep red cells incubated with culture supernatant from the transgenic hybrids and fluoresceinated (FITC) anti-mouse μ . Figure 6D is results using sheep red cells incubated with hybrid culture supernatant and fluoresceinated (FITC) anti-mouse kappa.

In Figures 6 and 8 fluorescence intensity of a fixed number of cells is plotted against cell number. In Figure 7 fluorescence intensity of a fixed number of cells is

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plotted against scatter, with each dot representing a cell.

A shift of the profiles to the right (increased fluorescence) denotes a positive stain that can only be seen for antibodies containing human mu heavy chains and mouse kappa light chains but not for antibodies containing mouse mu heavy chains or mouse gamma heavy chains (not shown).

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Table 1Antibody in the body fluids of IgA2, lambda 1-mice

Mouse	<u>IgA2 anti NP Ab</u>			<u>Total kappa bearing Ab</u>	
	Serum	Milk	Colostrum	Milk	Colostrum
TG1	10	0.6	2.1	960	600
TG2	6.3	0.56	1.4	1000	420
TG3	11.3	1.3	ND	735	ND
TG4	7.3	0.8	1.4	780	660
TG5	30	7.6	10.0	1250	ND
TG6	34.6	5.0	10.0	500	600
TG7	6.3	0.64	0.93	780	600
Control	0	0	0	1136	660

ND, not determined

All concentration in ug/ml

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CLAIMS

1. A method of producing an immunoglobulin, comprising obtaining immunoglobulin from cells or body fluid of a transgenic animal which has had inserted into its germline genetic material that encodes for at least part of an immunoglobulin of foreign origin or that can rearrange to encode a repertoire of immunoglobulins.
2. A method according to claim 1, wherein polyclonal antiserum comprising the immunoglobulin is obtained from the animal.
3. A method according to claim 1, wherein monoclonal antibody comprising the immunoglobulin is produced using cells obtained from the animal.
4. A method according to claim 1, wherein the immunoglobulin is produced in a body fluid or secretion of the animal.
5. A method according to claim 1, wherein the immunoglobulin is produced in vitro from cells obtained from the animal.
6. A method according to any one of the preceding claims, wherein the inserted genetic material encodes substantially the entire species specific regions of an immunoglobulin, and foreign immunoglobulin to a particular antigen is produced in response to subsequent introduction of the antigen to the animal.

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7. A method according to any one of the preceding claims, wherein the inserted genetic material is of human origin.
8. A method according to any one of the preceding claims, wherein the inserted genetic material encodes an entire immunoglobulin.
9. A method according to any one of the preceding claims wherein the inserted genetic material comprises a plasmid or cosmid; multiple plasmids or cosmids; a yeast artificial chromosome; or a mammalian chromosome fragment.
10. A method according to any one of the preceding claims, wherein the genetic material is inserted by injection or other procedures into fertilised eggs or embryonic stem cells.
11. A method according to any one of the preceding claims, wherein the animal initially does not carry genetic material encoding immunoglobulin constant regions.
12. An immunoglobulin of foreign origin produced by the method of any one of the preceding claims.
13. A transgenic animal, particularly non-human mammal, which has had inserted into its germline genetic material that encodes for at least part of an immunoglobulin of foreign origin or that can rearrange to encode a repertoire of immunoglobulins.

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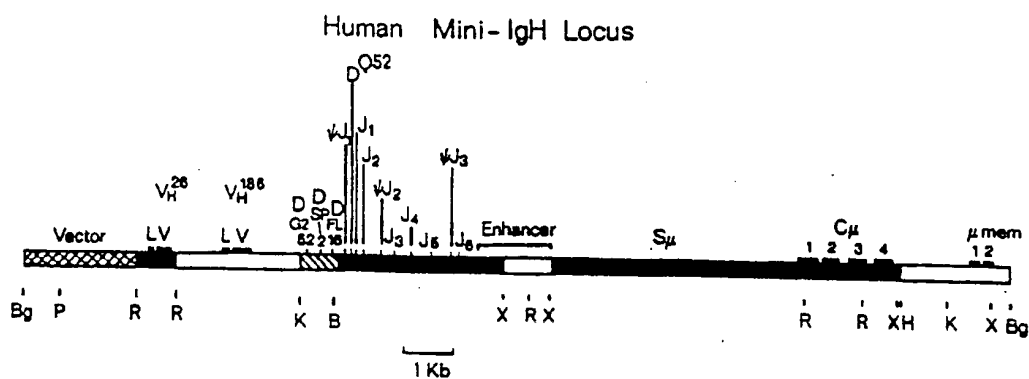
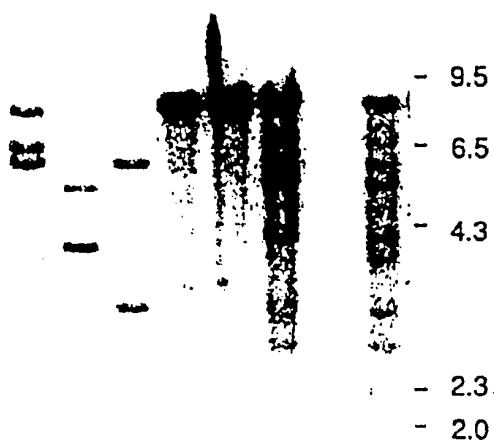


Fig. 1

Hybrids

35.1
22.18
16.20

Tail
Liver
Spleen
Control tail
Thymus



EcoRI

Fig. 2

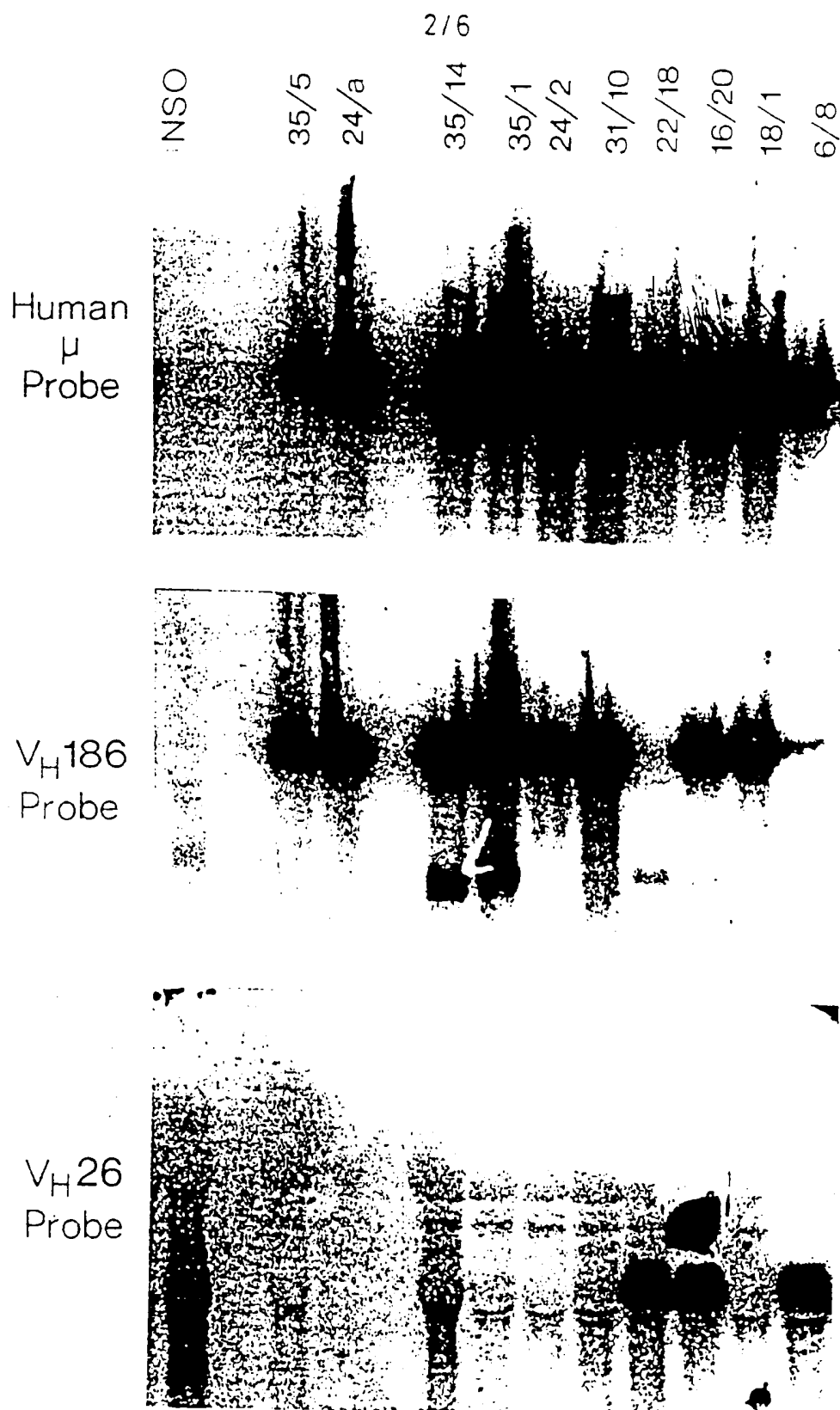


Fig. 3

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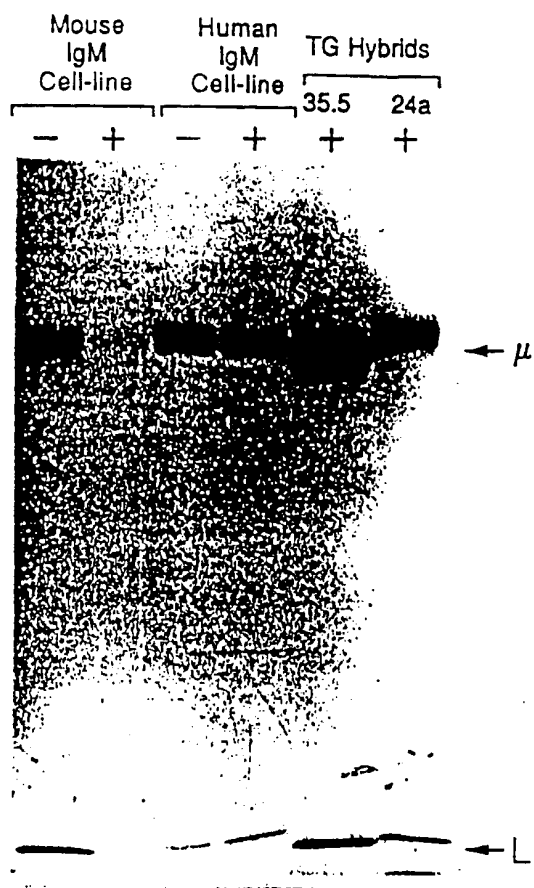


Fig. 4

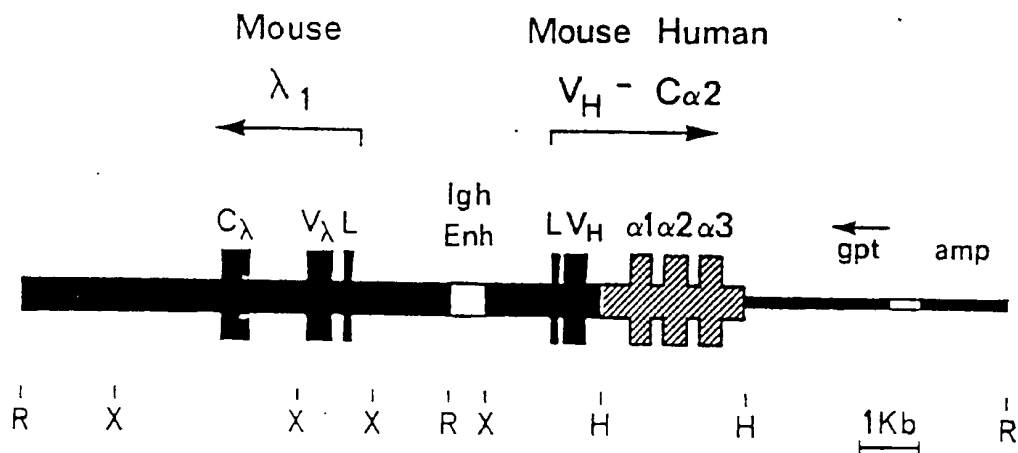


Fig. 5

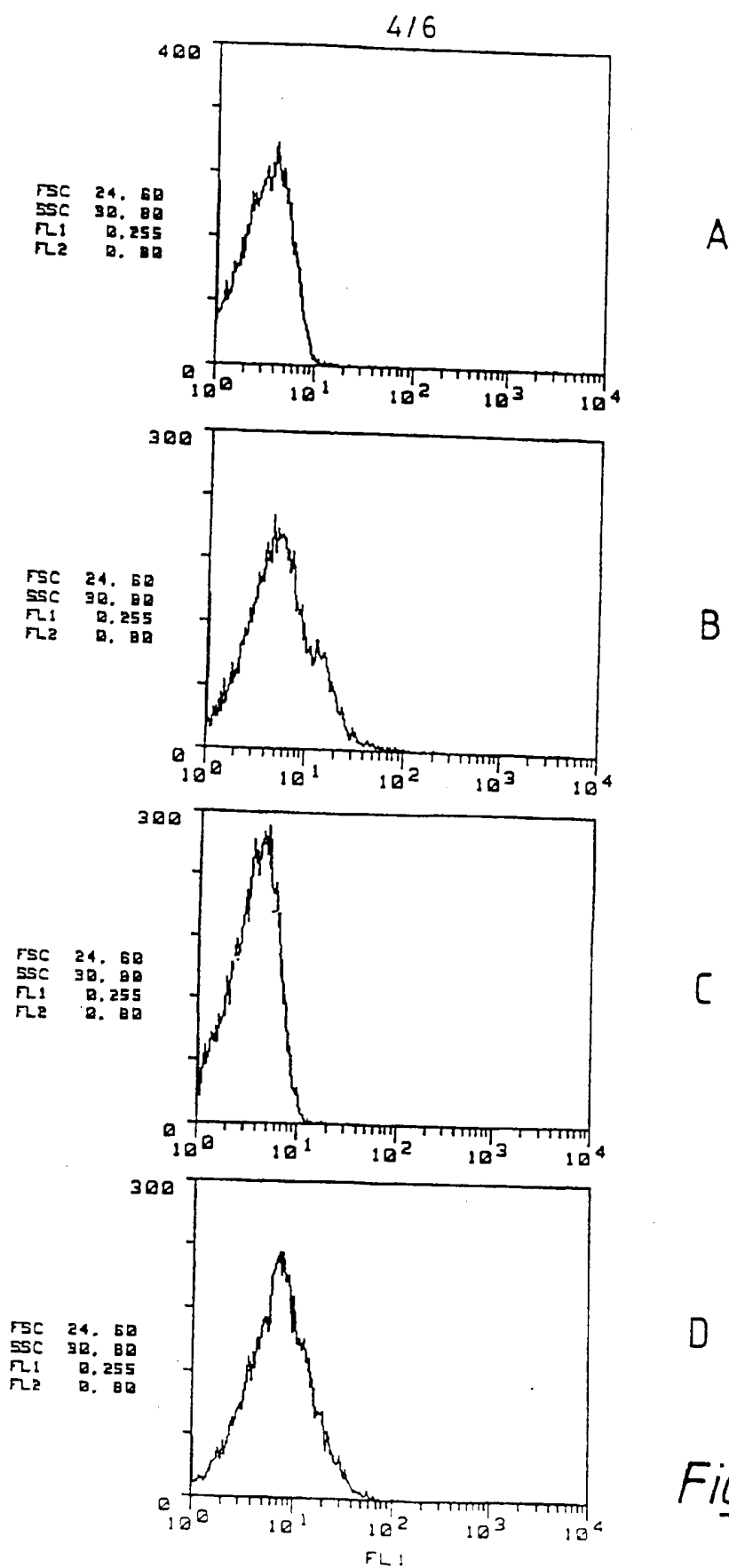
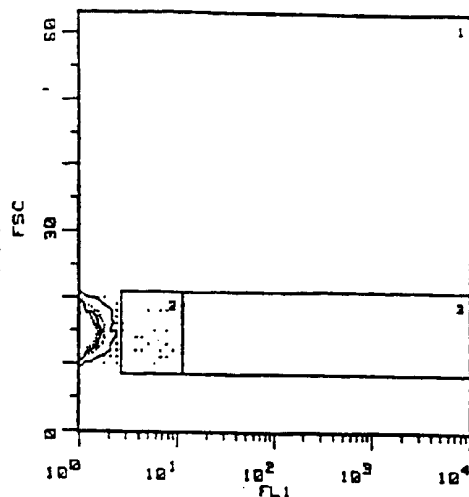


Fig.6

5/6

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SSC 0.255
FL1 0.255
FL2 0.255

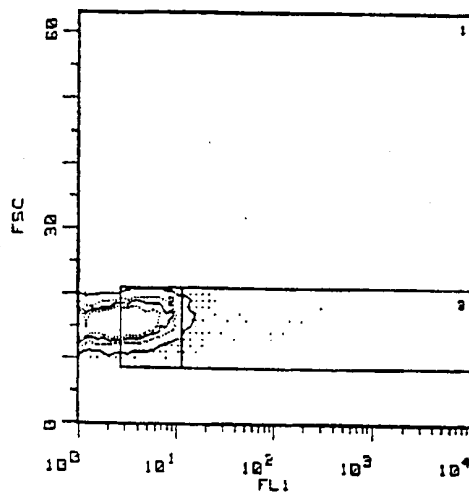
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30
55
80



A

FSC 0.255
SSC 0.255
FL1 0.255
FL2 0.255

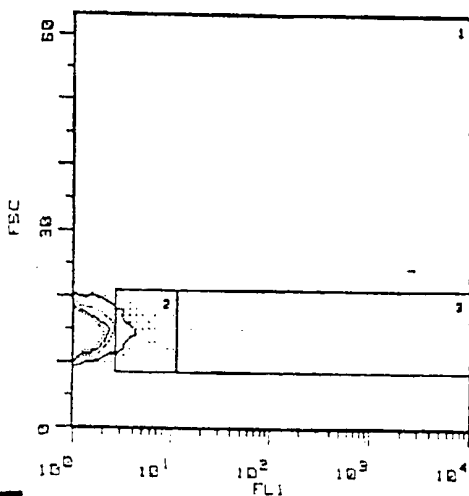
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B

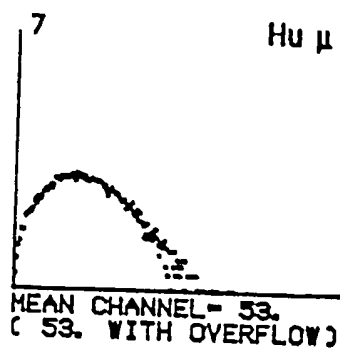
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55
80

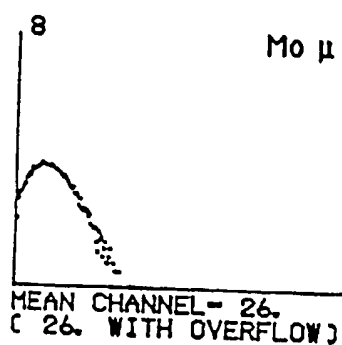


C

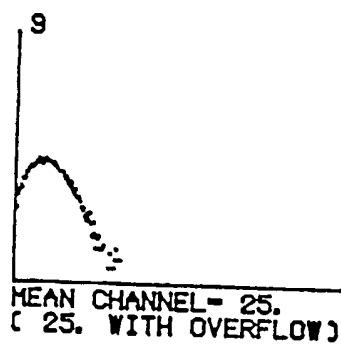
6/6



B



C

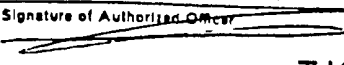


A

Fig. 8

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/01207

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 P 21/08, C 12 N 15/85, 5/10, 15/13		
II. FIELDS SEARCHED		
Classification System Minimum Documentation Searched *		Classification Symbols
IPC5	C 12 N; C 12 P	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched *		
III. DOCUMENTS CONSIDERED TO BE RELEVANT *		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
P, X	WO, A1, 88/10118 (BIOGEN N.V.) 29 December 1988, see page 5, line 28 page 4, line 20, example 4 and claim 2 page 13 lines 20-21	1-4, 7, 8- 10, 12, 13
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X	TIBTECH, Vol. 5, 1987, A.J. Clark et	1-13
Y	al: "Pharmaceuticals from transgenic livestock ", see page 20 - page 24	1-13
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X	TIBTECH, Vol. 5, 1987, R.B. Church: "Embryo	1-13
Y	manipulation and gene transfer in domestic animals ", see page 13 - page 19	1-13
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* Special categories of cited documents: ** "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such docu- ments, such combination being obvious to a person skilled in the art. "A" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 28th December 1989		Date of Mailing of this International Search Report 25. 01. 90
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer  T.K. WILLIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
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The members are as contained in the European Patent Office EPP file on 08/11/89
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